

# Toxin Production by *Campylobacter* spp.

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## INTRODUCTION

The identification and characterization of virulence factors is a major activity of microbiological research. Knowledge on the nature, regulation, and mechanism of action of virulence factors is indispensable for prevention and treatment of infectious diseases. *Campylobacter jejuni* is the most common bacterial cause of acute infective diarrhea in humans in many developed countries, and is isolated at a high frequency from young children, both healthy and diseased, in developing countries. Two types of diarrhea are observed with *C. jejuni* infections: inflammatory diarrhea, with fever and slimy, often bloody stools containing leukocytes; and noninflammatory diarrhea, with watery stools and the absence of leukocytes and blood. Much effort has been invested to elucidate the pathogenic mechanisms of *C. jejuni* and the closely related, less common *C. coli*. By analogy to other enteropathogens and considering the typical motility of *C. jejuni* and *C. coli*, four major virulence properties were recognized: motility, adherence, invasion, and toxin production (117).

The role of motility in the pathogenesis of *C. jejuni* is now well established (1, 13, 83, 85, 117). Motility is not only required for the bacteria to reach the attachment sites but is also required for their penetration into intestinal cells, although the exact role of flagella in this process has not been defined. Adherence of bacteria to the epithelial surface is probably an important determinant for colonization and may increase the local concentration of secreted bacterial products. Adhesion has been studied extensively in vitro, but specific adhesins, on

the flagella or on the bacterial body, have not been identified. The presence or absence of the recently discovered fimbrial structures on the surface of the bacteria did not influence adherence as measured in vitro, but was significant for colonization in rabbit ileal loops (26). Invasion, i.e., adherence followed by penetration, has been shown in vivo and in vitro (14, 24, 37, 61, 62), and putative factors required for invasion have been identified (24, 31, 60, 77, 118; see also reference 51 for a general review of the pathogenesis of *Campylobacter* spp.). However, invasion levels as detected in vitro are normally low: less than 1% of the applied bacteria invade a monolayer of cells in culture (14, 118), and efficient intracellular killing of bacteria takes place (23). Therefore, toxins have been considered important factors for the pathogenesis of *Campylobacter* enteritis.

The characterization of toxin production by *Campylobacter* spp. has been a slow process. Although the production of several toxins has been reported, their mechanism of action and their importance in disease remains unclear. Some groups have failed to detect any toxins produced by *Campylobacter* spp. This has led to a confusing discussion regarding the very existence of *Campylobacter* exotoxins and the relevance thereof.

This review attempts to summarize current knowledge of proteinaceous toxins produced by *Campylobacter* spp. An attempt has been made to classify the described toxins. The possible relevance to virulence will be discussed. The few cases where toxin production by *Campylobacter* species other than *C. jejuni* and *C. coli* has been described are included. Since several published studies compare *Campylobacter* toxins with known toxins from pathogenic enterobacteria, the major modes of action of the latter are briefly summarized here. Proteinaceous toxins that are relevant in the context of enteropathogenicity

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can be classified into two classes depending on their primary mode of action: enterotoxins and cytotoxins.

### Enterotoxins

Enterotoxins are defined here as secreted proteins with a capacity to bind to a cellular receptor, enter the cell, and elevate intracellular cyclic AMP (cAMP) levels. The prototypes of enterotoxin (also called cytotoxic toxin) are *Vibrio cholerae* toxin (CT) and the closely related *Escherichia coli* heat-labile toxin (LT) (108). CT and LT comprise two subunits: the larger A subunit, which has enzymatic activity, and the smaller B subunit, which is present as a pentamer and contributes the binding activity to a receptor. After binding to the cellular receptor ganglioside GM<sub>1</sub>, subunit A is transported into the cell and, after proteolytic activation, deregulates the cellular adenylate cyclase regulatory system by ADP-ribosylation. As a result, the intracellular cAMP levels rise, and ion flux changes cause excess secretion of fluid, which results in watery diarrhea. The primary structures of the CT and LT subunits have considerable homology, and the toxins share immunological epitopes. The B subunits are immunodominant. Enterotoxin activity can be demonstrated in vitro by the elongation of exposed cultured Chinese hamster ovary (CHO) cells or rounding of mouse adrenal tumor cells (Y-1) or by measurement of intracellular cAMP levels in exposed cells. A method independent of cultured cells is an enzyme-linked immunosorbent assay (ELISA). GM<sub>1</sub> gangliosides are fixed to the solid phase to bind enterotoxin, which can then be detected by specific antisera. In the alternative sandwich ELISA method, ganglioside binding is not used; instead, the solid phase consists of F(ab')<sub>2</sub> fragments of anti-CT or anti-LT antibodies to bind enterotoxin, and detection occurs after the binding of antiserum to CT or LT. Both methods display high sensitivity and specificity. In vivo assays to detect enterotoxin activity include the rabbit or rat ileal loop test (RILT) (32, 84), or the removable intestinal tie adult rabbit diarrhea (RITARD) model (109).

### Cytotoxins

Cytotoxins are defined as proteins that kill target cells. Cytotoxins can act intracellularly or form pores in the cells. Cytotoxins with intracellular activity generally bind to the cells and are processed before they reach the cell cytoplasm. Different mechanisms of toxicity exist, of which two predominate: inhibition of cellular protein synthesis and inhibition of actin filament formation. Examples of the first type of cytotoxin are *Shigella dysenteriae* toxin and the related *E. coli* Shiga-like toxin (Stx) (the nomenclature is that of reference 17), also known as verotoxin (reviewed in reference 87). The two toxins are closely related and contain two subunits, the A subunit with enzymatic activity and a pentamer of B subunits (89). The B subunits bind to the sphingoglycolipid receptor Gb<sub>3</sub>. Upon uptake of the holotoxin, the subunit A is proteolytically cleaved. This activated A fragment has depurinating activity, similar to plant-derived toxins like ricin, and inactivates ribosomes by depurination of a nucleotide in the 28S rRNA (27, 99). The resulting inhibition of protein synthesis leads to cell death. This is easily measured in sensitive cells like African green monkey kidney (Vero) cells and human tumor epithelial (HeLa) cells. CHO cells are insensitive due to the lack of receptor, and this makes it easy to identify Stx.

Examples of a cytotoxin inhibiting actin filament formation include *Clostridium difficile* toxins A and B (67). Toxin A is also referred to as the enterotoxin of *C. difficile*, because it damages the intestinal mucosa and causes diarrhea (115). Toxin B is the

more potent cytotoxin of the two. Both toxins cause disruption of the actin cytoskeleton by covalently modifying Rho proteins (25, 47). Antiserum against toxin A and B completely neutralizes these toxins, but antitoxin A does not neutralize toxin B and vice versa (114). The toxins cause rounding of CHO cells or any other epithelial cell line.

The second major mechanism of cytotoxin action is the formation of pores in target membranes. Such cytolysins can often be detected by virtue of their lytic activity on erythrocytes (11, 12, 100, 119), for which they are also known as hemolysins. However, the true pathogenic relevance of pore-forming toxins lies in their toxicity toward nucleated cells (12). Pore-forming toxins elicit a broad spectrum of secondary reactions in nucleated cells, including cytokine release, cytoskeleton dysfunction, secretion of granule constituents, and generation of lipid mediators. These reactions can cause profound local and distant effects in host tissues (12). During infection, lysis of erythrocytes may be a mechanism for iron acquisition, but it may also only be fortuitous, with other cell types being the major targets for the toxin in vivo. In this respect, killing of leukocytes and granulocytes or macrophages may hamper the immune response of the host. Examples of well-characterized pore-forming hemolysins include *E. coli* alpha-hemolysin (HlyA) and the other, related, RTX toxins, which all contain repeats in their primary structure (RTX stands for "repeat in toxin") (11, 16, 119). However, *E. coli* HlyA is not produced by most intestinal strains and is a virulence factor mainly for extraintestinal infections (10). In contrast, the hemolysin produced by *V. cholerae* El Tor is a possible candidate for the causative agent of diarrhea in CT-negative strains (43) and also acts by forming pores in the membrane of the target cell (122). The protein is secreted by an unknown mechanism and is proteolytically activated (121). Direct evidence for a pore-forming toxin that causes diarrhea is available for *Vibrio parahaemolyticus* thermostable direct hemolysin, which induces intestinal chloride secretion and diarrhea in a rabbit model whereas isogenic mutants fail to do so (86, 98).

### ENTEROTOXIN PRODUCTION BY *CAMPYLOBACTER* SPP.

Enterotoxin activity of *C. jejuni* was first documented in 1983 (102). Culture supernatants of a *C. jejuni* strain caused intraluminal fluid secretion in the rat RILT model. Elongation of CHO cells paralleled by increased intracellular cAMP levels was observed in vitro. The detection of enterotoxin activity in culture supernatants of *C. jejuni* was confirmed by other groups (35, 44, 55, 71, 72), and the toxin(s) was abbreviated CJT for *C. jejuni* toxin (which will be used here), CYTON or CTON for cytotoxic toxin, or CCT for *Campylobacter* cytotoxic toxin. The reported neutralization of CJT with anti-LT and anti-CT antibodies (35, 55, 102) suggested an immunological similarity to *V. cholerae* CT and *E. coli* LT.

These early reports on enterotoxin production were soon followed by contradicting evidence showing that all tested sonicates of *C. jejuni* and *C. coli* were nonenterotoxic toward CHO or Y-1 cells and did not cause fluid secretion in the rabbit RILT model (116). However, the RILT was also described to be negative for rabbits but positive for rats in the original publication and in others (55, 102, 104). At the same time, it was shown that sonicates of strains expressing enterotoxin in the supernatant are inactive, possibly due to the release of proteases during bacterial lysis (55, 72). More disturbing was the fact that a CT DNA probe failed to hybridize to a Southern blot containing *Campylobacter* DNA (90). The strong homology between the CT and LT genes and the immunological

cross-reactivity between their gene products and CJT would suggest some detectable genetic homology between CT/LT and CJT. With the use of two oligonucleotides specific for LT subunits A and B, respectively, no hybridization was detected between *C. jejuni* DNA and subunit A and only one of five enterotoxigenic strains hybridized with the oligonucleotide for subunit B (5). This hybridization signal has not led to the cloning of the CJT gene. Although much work was carried out to isolate and characterize the enterotoxigenic protein(s) and to correlate its activity with pathogenicity, a number of investigators questioned the expression of a CT/LT-like enterotoxin by *C. jejuni* and *C. coli*.

Nevertheless, the immunological properties of CJT were compared with CT and LT by slide gel immunodiffusion, ELISA, Ouchterlony, and immunoblot (Western blot) techniques (55, 57, 72, 73, 111); CJT reacted with antiserum against LT or CT, and CT reacted weakly with anti-CJT antiserum. The immunological cross-reaction of the proposed B subunit of CJT was stronger with LT than with CT, suggesting a closer antigenic similarity between CJT and LT (57). In a study with serum from young patients and carriers of *C. coli* from the Central African Republic, antibodies cross-reacting with both A and B subunits of CT were detected at high titers, implying that immunogens homologous to both subunits are expressed during carriage of and infection with *C. coli* (69). Compared to cholera patients, however, weak titers were found in the sera of *C. jejuni* patients in an ELISA with LT as antigen (41).

#### Detection and Purification

Enterotoxin activity of *C. jejuni* culture supernatants was detected in vitro by observing the elongation of CHO cells and rounding of Y-1 cells. The latter are reportedly less sensitive (44) but have the advantage that they are insensitive to the cytolethal distending toxin (CDT) of *Campylobacter* spp. (46; see below). Maximal enterotoxin activity was found in cultures approaching the stationary phase (22, 55). Culture conditions such as medium supplements or growth temperature influence the yields (35, 55). IsoVitalX (1%) is often added to the culture medium to increase enterotoxin production. The component responsible for this effect is probably ferric iron, and the addition of ferric chloride to culture medium increases both the amount of enterotoxin produced and the detection of enterotoxin-positive strains (74). Culture filtrates become more strongly positive after polymyxin B treatment (44, 50, 55, 102), perhaps because the enterotoxin is located in the periplasm (18, 50). In contrast, prolonged incubation with highly concentrated polymyxin B decreases the yield of CJT (72, 73), just as bacterial lysis by sonication does, probably by causing the release of proteases (55, 72, 116).

Enterotoxin activity of *C. jejuni* was also demonstrated by ELISA, making use of the cross-reactivity to anti-CT or anti-LT antisera. In an early study (55), two ELISAs were compared: a GM<sub>1</sub> ELISA in which gangliosides were bound to the solid phase and, subsequently, bound CJT was detected with antiserum to LT; and an ELISA with anti-LT F(ab')<sub>2</sub> fragments as the solid phase to bind CJT, which was then detected with an anti-LT antiserum (sandwich LT ELISA). Concentrated culture supernatants that caused fluid secretion in the RILT model were also positive in the two ELISAs and in the CHO cell assay, with the GM<sub>1</sub> ELISA being slightly more sensitive (55). The opposite is true for nonconcentrated supernatants: the GM<sub>1</sub> ELISA resulted in low titers and was less sensitive than the CHO cell assay (66). A sandwich ELISA based on CT was positive in cases where the GM<sub>1</sub>-ELISA was negative and was at least 10 times more sensitive than the

CHO assay (73). In conclusion, the sandwich CT ELISA and the GM<sub>1</sub> ELISA with concentrated culture filtrates are more sensitive than the CHO cell assay in detection of CJT.

Attempts have been made to purify and biochemically characterize CJT. The enterotoxin activity could be concentrated by ammonium sulfate precipitation and partly purified on a galactose-agarose column (galactose is a component of ganglioside) (56, 57) or by affinity chromatography with gangliosides (21) or anti-CT antibodies (72, 73). One early study described the purification of CJT by affinity chromatography with anti-CT antibodies, which resulted in a toxic fraction (as determined by Y-1 cell rounding and rabbit RILT) that contained a single 70-kDa band (pI 9.0) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). No subunits were detected under stringent denaturing conditions (72, 73). A different purification strategy, involving galactose affinity chromatography, contradicted these results, and it was speculated that CJT contains functional subunits (57): a highly concentrated toxic fraction (as determined with CHO cells) was obtained that lost toxicity for CHO cells or ileal loops after further purification (56, 57). This fraction was proposed to contain purified B subunit as it gave a positive reaction in the GM<sub>1</sub> ELISA, but multiple bands were visible after SDS-PAGE, so that a molecular weight could not be addressed to the B subunit (57). This became possible when Daikoku et al. applied a similar purification strategy of ammonium sulfate precipitation, gel filtration, and ganglioside affinity column chromatography (21). The resulting toxic fraction (reacting positively in a GM<sub>1</sub> ELISA) contained three proteins of 68, 54, and 43 kDa on SDS-PAGE and caused elevated cAMP levels in HeLa cells (21). Further purification on an affinity column with anti-CT antibodies resulted in a pure 68-kDa band that was positive in a GM<sub>1</sub> ELISA. Instead, if the original toxic fraction was purified further by ganglioside affinity chromatography, the 68- and 54-kDa bands were recovered. Unfortunately, the toxicity of these purified preparations was not tested. Reconciling these results, it seems likely that a single 68-kDa (or 70-kDa) protein is able to bind to gangliosides, cross-reacts with antiserum against LT and CT, is toxic for CHO and Y-1 cells, and causes fluid secretion in ileal loops. The reported loss of toxicity during purification of a proposed B subunit (57) could be due to inactivation. Toxic properties of the copurified 54- and 43-kDa proteins were not shown.

Completely different results are described in a more recent paper (19): *C. jejuni* culture filtrates were concentrated by ammonium sulfate precipitation, analyzed by SDS-PAGE, and immunoblotted to be visualized with anti-CT antiserum. Under native conditions, this resulted in a single band, which migrated similarly to control CT. Denaturing electrophoretic conditions resulted in a high-molecular-mass band (>90 kDa) and a 50-kDa band on the immunoblot. The culture filtrates gave a positive reaction in a direct antigen ELISA, in which the filtrates were used to coat the plates and detected with anti-CT antiserum, but a negative reaction in a GM<sub>1</sub> ELISA, and inconsistent results were obtained with CHO cell assays. Since the filtrates caused no fluid accumulation in rat ileal loops (19), the enterotoxigenicity of these proteins is questionable.

#### Incidence of Enterotoxin Production

The reported frequency of enterotoxin production varies widely among isolates (Table 1). The highest frequencies were found in clinical isolates from Belgium (28, 35), the United States (74), South Africa (15), and Mexico (102) (Table 1). At the other extreme, no enterotoxigenic strains were detected in clinical isolates from the United States by two different groups

TABLE 1. Incidence of enterotoxin production among strains of *C. jejuni* and *C. coli*

No. and origin of strains	% Enterotoxin positive	Method of detection <sup>a</sup>	Reference
25 clinical, Belgium	100	CHO	35
62 clinical, United States	94	GM <sub>1</sub> ELISA <sup>b</sup>	74
22 clinical, South Africa	77	Y-1	15
44 clinical, Belgium	75	CHO	28
32 clinical, Mexico	65	CHO, RILT	102
80 clinical, Algeria	65	CHO	6
12 clinical, diverse origin	50	CHO, GM <sub>1</sub> ELISA, RILT	47
316 clinical, Canada	48	CHO, Y-1	45
44 clinical, Costa Rica	47	Y-1	33
372 clinical, diverse origin	45	CHO	65
39 clinical, United States	36	GM <sub>1</sub> ELISA	59
202 clinical, Sweden	32/19 <sup>c</sup>	CHO/GM <sub>1</sub> ELISA <sup>c</sup>	66
22 clinical, India	32	CHO, GM <sub>1</sub> ELISA	71
22 clinical, United States	0	CHO, GM <sub>1</sub> ELISA	93
15 clinical, United States	0	CHO, GM <sub>1</sub> ELISA	63
47 carriers, India	12	CHO, GM <sub>1</sub> ELISA	71
30 carriers, Algeria	60	CHO	6
6 carriers, Mexico	16	CHO, RILT	102
8 carriers, Mexico	0	CHO	101
77 carriers, India	0	CHO, GM <sub>1</sub> ELISA, RILT	58
107 chicken	59	CHO	65
16 chicken	31/100 <sup>d</sup>	RILT	104
35 chicken	34	Y-1	33
28 chicken	32/14 <sup>c</sup>	CHO/GM <sub>1</sub> ELISA <sup>c</sup>	65
67 chicken/animal	0	CHO	76
17 horse	76	CHO	52

<sup>a</sup> CHO, elongation of Chinese hamster ovary cells; GM<sub>1</sub>ELISA, ELISA with ganglioside GM<sub>1</sub> as the solid phase and antiserum against enterotoxin CT or LT as primary antiserum; Y-1, rounding of mouse adrenal tumor cells; RILT, fluid accumulation in the rat ileal loop test.

<sup>b</sup> The primary antiserum used was homologous serum against enterotoxin of *C. jejuni*.

<sup>c</sup> Different results were obtained by the two methods applied.

<sup>d</sup> All 16 strains became enterotoxigenic after passage through the rat gut.

(63, 93). These conflicting results reflect differences in methodology and in parameters such as the number of passages and strain storage (5, 22, 33), culture conditions, and polymyxin B treatment.

The frequency of enterotoxin production in human isolates was compared to that in animal isolates. In strains isolated from poultry and other animals in the United Kingdom, no enterotoxin production was detected (76). In that study, one strain earlier described as enterotoxin positive was included, but its enterotoxin activity could not be confirmed (76). In contrast to these findings, enterotoxigenic *Campylobacter* strains were isolated from Swedish chickens at the same frequency as from humans (66).

A possible correlation of enterotoxin production with other epidemiological features has been addressed, with disappointing results. No correlation was found between enterotoxin activity and prevalent Lior or Penner serotypes (66) or certain biotypes (28). Enterotoxin activity is not linked to the presence of tetracycline resistance plasmids (113).

In comparison, *C. jejuni* has a higher incidence of enterotoxin production than *C. coli* (6, 65). Enterotoxin activity has been demonstrated in non-*jejuni/coli* *Campylobacter* species: in one study, two human isolates and two of four nonhuman isolates of *C. lari* expressed enterotoxin as determined by CHO elongation (45), but in contrast, all eight *C. lari* isolates from

pigs were enterotoxin negative in another study (65). Stable expression of enterotoxin by a *C. hyointestinalis* strain was reported once (46).

In conclusion, the methods and results of these studies vary so much that no general statement on the incidence of enterotoxin production by *Campylobacter* spp. can be made. On the one hand, storage and the number of passages in the laboratory can decrease enterotoxin production by clinical isolates; on the other hand, two or three passages through the gut of rats induce expression in every enterotoxin-negative strain tested (104). This indicates that the expression of enterotoxin is regulated, like many other bacterial virulence factors. Only when genetic probes for CJT become available can the percentage of *Campylobacter* strains that have the capability to produce enterotoxin be assessed.

### Relevance to Disease

It has been suggested that enterotoxin production results in the watery type of diarrhea, as opposed to inflammatory bloody diarrhea due to cytotoxin production and invasion (117). If this were a reflection of strain differences, enterotoxigenic strains would be predominant in patients suffering from watery diarrhea. Such a correlation was found in some but not all studies. In Costa Rica, significantly more enterotoxin-positive strains were isolated from patients with watery diarrhea than from patients with inflammatory diarrhea (33). In Mexico, patients infected with toxigenic strains more often suffered from watery and not bloody diarrhea, whereas asymptomatic carriers were more often infected with nontoxigenic strains (101, 102). Enterotoxin-negative strains were significantly more invasive in vitro (64). Finally, in another study, all six strains isolated from patients in the United States with watery diarrhea caused fluid secretion in ligated ileal loops and displayed enterotoxin activity, whereas all eight isolates from asymptomatic carriers were negative (58). However, in studies in India (71) and Algeria (6), enterotoxigenic strains were isolated from carriers as well (Table 1). The serum of infected African children, carriers, and controls were positive for antibodies to CT (70), although to variable extents. In addition, no clinical correlation with enterotoxin activity was seen in a study in Brussels (28). Since travelers in developing countries usually suffer from inflammatory diarrhea after *Campylobacter* infection, the difference between watery and inflammatory diarrhea is more likely to be determined by the host than by the bacteria (107).

Enterotoxin of *V. cholerae* and *E. coli* is immunogenic during infection, and so it was expected that exposure to enterotoxigenic strains of *C. jejuni* would result in seroconversion. In the studies in Mexico, serum antibodies against CJT were detected mainly in patients infected with enterotoxigenic strains (102). Antibodies against CJT have reportedly been demonstrated in infected children in Mexico, Japan, and Africa (41, 69, 101). However, in the combined U.S.-Thai study, only 1 of 64 patients seroconverted to the enterotoxin, as determined with LT as the antigen (93, 94). In this study, the absence of a humoral response against CJT was explained by a different mechanism of pathogenesis. Because most patients had leukocytes in their stools, these strains were considered invasive and/or cytotoxic; enterotoxin production was not detected (93).

Seroconversion is not a good indicator of clinical relevance (106). The absence of a humoral response against a certain bacterial factor indicates that the factor was not expressed in vivo or that it was not immunogenic or not accessible to the immune system of the host. Such immunologically "silent" factors may or may not be important for pathogenesis. Since LT and CT are immunogenic during infection, it is unlikely

that related CJT is immunologically silent. If, on the other hand, seroconversion does occur, it shows that the factor was expressed and immunogenic, but it is not proof of its relevance to the disease. Finally, the high incidence of *E. coli* and *V. cholerae* expressing enterotoxin in developing countries masks the immune status to CJT of patients from these countries (70).

### Animal Models of Toxicity

Fluid secretion can be induced in ileal loops of rats by (concentrated) culture filtrates or viable bacteria of toxigenic *Campylobacter* spp. (30, 55, 102, 104). All tested *C. jejuni* strains that were negative in this RILT model became positive when the bacteria were recovered from the original loop and tested in subsequent ileal loops (104). Even coccoid, nonculturable forms of *C. jejuni* could be recovered from gut loops and became enterotoxic after two or three consecutive passages in rats (103). The fluid secretion could be inhibited by pretreatment of the culture filtrate at a nonpermissive temperature or low pH or by preincubation with cholera antitoxin in a concentration-dependent way (102, 104). Moreover, preimmunization of rats with subunit B of LT prevented the induction of fluid secretion by CJT (57).

Analysis of the composition of the fluid secreted by *C. jejuni*-infected loops showed that this fluid had some similarities to the fluid secreted as a result of CT, e.g., increased bicarbonate concentration (30) and elevated cAMP levels in tissue homogenates (29). Infection with an enterotoxin-negative strain also resulted in fluid secretion, but at lower levels compared with those produced by enterotoxigenic strains, whereas a nonmotile, noninvasive mutant did not result in fluid secretion or elevated cAMP levels (29, 30). In contrast to loops treated with CT, tissue damage was observed, as indicated by increased hemoglobin levels (30). The role of CJT in these pathological effects was questioned; the ileal loops were inoculated with viable, invasive bacteria that also expressed cytotoxin(s). The increased cAMP level was explained differently: the loop fluid in infected loops contained prostaglandin  $E_2$ , an inflammatory mediator that could cause elevated cAMP levels in cultured cells (29). No enterotoxin was detected in loop fluid, as determined by GM<sub>1</sub> ELISA (30). If CJT was expressed at all, it may have remained bound to the target cells, and in this respect it may differ from CT. Another difference between CJT and CT is the ineffectiveness of CJT in ileal loops in rabbits, with the exception of the results described by McCardell et al. (72, 73). Furthermore, the GM<sub>1</sub> ganglioside specificity of CT and CJT differ as determined in vitro (111).

An alternative, calcium-dependent mechanism was proposed for the fluid accumulation in ileal loops (48, 49). Viable bacteria of an enterotoxigenic strain were inoculated in the rat RILT model with and without modulators of calcium channels, calmodulin and protein kinase C (PKC). The results indicate that the enterotoxigenic strains cause an increased influx of  $Ca^{2+}$  into the enterocytes, which activates PKC, without the involvement of calmodulin (49). In response to the increased intracellular  $Ca^{2+}$  level, PKC functions as a second messenger to increase ileal electrolyte secretion, possibly by decreasing the activity of the  $Na^+, K^+$  ATPase pump. This explanation for the fluid secretion was affirmed when the loop segments were tested in an Ussing chamber: a decreased  $Na^+, K^+$  ATPase activity, combined with a decreased absorption of sugars and a net secretion of NaCl, was observed (41). The role of host inflammatory mediators, such as prostaglandins or leukotrienes, was not investigated. The experiments did not include a control of an enterotoxin-negative strain or a purified CJT

preparation, and so it cannot be concluded that the observed effects were (solely) due to the enterotoxin.

### The Controversy of Enterotoxin

Despite the work summarized above, it remains controversial whether *Campylobacter* spp. produce CT-like enterotoxin and, if so, what its importance is for pathogenicity. The major argument in the debate is the early observation that there is no homology at the DNA level between the genes coding for CJT and CT (90, 94), despite the immunological cross-reactivity in vitro and in vivo between CJT and CT or LT. In an attempt to trace the structural gene for CJT, a degenerate oligonucleotide was developed that coded for the ganglioside-binding site of CT and LT, which might be homologous to the functional domain in CJT. This oligonucleotide hybridized to a *C. jejuni* genomic fragment (15), and this result was confirmed by another group (63); however, attempts to amplify the enterotoxin gene by PCR with this oligonucleotide and a panel of other primers failed (63). It must be assumed that the degenerate oligonucleotide hybridized to irrelevant sequences.

As mentioned above, several groups were unable to detect any enterotoxin activity in *Campylobacter* strains (63, 76, 93, 116). Technical explanations for these negative results were given where applicable, but even in studies in which care was taken to promote enterotoxin production, the strains examined failed to do so (63, 94). Moreover, in other studies, the evidence for enterotoxin activity is not convincing, e.g., when only one technique was used. The CHO cell assay responds to the CDT cytotoxin as well; uninoculated, concentrated culture medium can contain compounds reactive in a GM<sub>1</sub> ELISA or immunoblot assay (63); fluid accumulation observed in the rat RILT model with inoculated viable organisms may be caused by invasion or cytotoxin production or both (30).

Still, the amount of circumstantial evidence that *C. jejuni* and *C. coli* express enterotoxic activity cannot be ignored, although this proposed enterotoxin is not as closely related to CT as was once assumed. For more convincing evidence, purified CJT (e.g., purified by the method in reference 21) should be tested in the RILT model, and if this results in fluid secretion, the mechanism of this secretion should be compared to that observed with viable organisms (29, 49). The identification of the structural gene for CJT would settle the argument. Specific antiserum against purified CJT protein has been produced (111), and this can be used to screen an expression gene library (cloned into a vector with a strong, regulated promoter) of an enterotoxigenic strain. Since virulence genes of *Campylobacter* may not be expressed in *E. coli*, screening could be done in an enterotoxin-negative *Campylobacter* strain. If this strategy is successful, a genetic analysis of CJT will answer the question of how this enterotoxin relates to LT and CT.

### CYTOTOXIN PRODUCTION BY *CAMPYLOBACTER* SPP.

#### Detection of Apparently Different Cytotoxins

The literature on *Campylobacter* cytotoxin production is confusing: different groups use different cell lines and bacterial culture conditions and strains, and little attempt has been made to compare the observed cytotoxic effects with those already described. On the basis of a weighed comparison of the data, the existence of six different cytotoxins is proposed: (i) a 70-kDa cytotoxin active on HeLa, CHO, and other cells but inactive on Vero cells; (ii) a cytotoxin active on Vero and HeLa cells; (iii) CDT; (iv) a cytotoxin neutralized by Stx antitoxin; (v) a cytotoxin(s) displaying hemolytic activity; and (vi) a hepatotoxin.

The criteria used here to classify the cytotoxins are cell specificity, which is reproducible and objective, and, when available, the molecular weight of the isolated toxin. The reported heat stability is not a reliable parameter and varies with the purity of the preparation. Cytotoxic activity is ascribed here to one toxin out of simplicity. However, the possibility that overall toxic activity is due to more than one factor must be considered.

**70-kDa cytotoxin.** An early report on the cytotoxicity of bacterial supernatants described a toxic activity for HeLa, HEp-2, and MRC cells but not for Vero or other animal cell lines (120). A later study by the same group described a molecular weight of more than 30,000 for the cytotoxin, and bacterial culture broth produced diarrhea in the RITARD model (91). The cytotoxin was heat sensitive, and expression was lost upon subculturing.

Possibly a similar or identical cytotoxin was described by Goossens et al. (36), who reported on a cytotoxin active on CHO cells which could not be neutralized by Stx antitoxin. Unfortunately, the sensitivity of Vero cells was not tested. However, in a study describing the Vero/HeLa cell cytotoxin (see below), Johnson and Lior (45) also detected a cytotoxin that was specific for HeLa and CHO cells and inactive on Vero cells, and they speculated that this was identical to the cytotoxin described by Goossens et al. Thus, this toxin is probably Vero inactive. A similar cytotoxin was detected by another group (75), who described a cytotoxin of 70 kDa that was toxic for CHO cells, although the observed heat stability and trypsin resistance of this cytotoxin were in contrast to other reports.

In accordance with the above data, Guerrant et al. (39) described a cytotoxin for HeLa and CHO cells, but not for Vero cells, with an estimated molecular weight of 50,000 to 70,000. The toxin was not neutralized by anti-*Clostridium* or Stx antitoxin and was heat and trypsin sensitive (38, 39). In another study (68), the cytotoxicity of a 68-kDa protein for CHO and INT407 cells was described. The cytotoxin was heat labile and trypsin sensitive, and a possible (glyco)protein receptor was suggested on chicken embryo fibroblast cells and INT407 cells. The cytotoxin described by Japanese researchers (22, 50, 81) was active on HeLa and CHO cells, heat labile, and trypsin sensitive. This cytotoxin may be the same 70-kDa toxin, but no neutralization studies or Vero cell specificity tests were performed to verify this similarity.

Two studies investigating the clinical significance of toxin production probably dealt with the same toxin: in one study (93), the CHO cell assay as described in reference 39 was used, so that presumably the identical cytotoxin was measured. In the other study, cytotoxicity to CHO cells could not be neutralized by Stx antitoxin (28).

The expression of cytotoxin was unstable and was lost upon subculturing (22, 91) or storage (22, 33) of the strains. An increase in the toxicity of filtered cultures was reported after polymyxin B treatment (38, 39, 44), but this was not always observed (33). The cytotoxin may be membrane associated and secreted: cytotoxic activity could be detected in the LPS fraction of a bacterial lysate (50). Solubilization experiments suggested that the protein was produced intracellularly in the early log phase, and released in the stationary phase (21, 22, 50).

In summary, a cytotoxin of approximately 70 kDa, which is heat labile and trypsin sensitive, is active against HeLa, CHO, HEp-2, and INT407 cells and inactive against Vero cells. The toxin cannot be neutralized by anti-Stx or anti-*Clostridium* antitoxin. The mechanism of action (intracellular versus membranolytic) is unknown.

**Vero/HeLa cell cytotoxin.** Fewer reports have been published on a cytotoxin that is active on Vero cells (33, 44, 45, 58). The toxin could not be neutralized with anti-*Clostridium* or Stx antitoxin. Vero cells were reported to be more sensitive than HeLa cells (44, 45).

In contrast, Klipstein et al. (58) found HeLa cells to be more sensitive than Vero cells. The authors credited this to a difference in the experimental protocol (58). In most instances, however, Vero cells were found to be more susceptible than HeLa cells (33, 44, 45), and in one study, MRC5 cells, which had not been tested by other groups, were even more sensitive (33). A strong cytotoxic effect on HeLa cells, weaker reaction on Vero cells, and very weak reaction on Y-1 cells was observed with bacterial sonicates after polymyxin B treatment of strains from Bangladesh (2). The mechanism of action of the Vero/HeLa cell cytotoxin is unknown.

The use of a tetrazolium dye to demonstrate cytotoxicity of polymyxin B-treated extracts of *C. jejuni* for HeLa and Vero cells was recently described (20). The dye assay was as sensitive as Trypan blue staining and could be easily quantified. However, it may be less sensitive than screening morphological changes of the cells, as described by Johnson and Lior (45) because culture supernatants did not produce cytotoxic activity in the dye test (20).

**Cytotolethal distending toxin.** CDT, produced by *Campylobacter* spp., was first described in 1988 by Johnson and Lior as CLDT (46). CHO, Vero, HeLa, and HEp-2 cells were sensitive and Y-1 cells were insensitive to its action. The toxin caused elevated intracellular cAMP levels and elongation of CHO cells, effects indistinguishable from those of enterotoxin activity after 24 h of incubation. However, after longer incubation (2 to 4 days), the cells showed extensive distension and death as determined by trypan blue sensitivity, which is in contrast to the effects seen with enterotoxin-treated cells (46). Differentiation between enterotoxin and CDT activity was obvious after 96 h. This cytotoxin is immunologically different from the Vero/HeLa cell cytotoxin, since antiserum raised against the latter did not neutralize CDT (46).

The morphological changes observed in another study suggest that CDT was detected on CHO cells whereas HeLa, Vero, and INT407 cells were insensitive (79, 80). However, these cell types became sensitive when newborn calf serum rather than fetal calf serum was used in the assay (79). A putative receptor for CDT was detected on CHO and HeLa cell membranes by ligand blotting, and a receptor-based ELISA was developed to detect CDT activity (4). This test is faster than the cytotoxic assay and is enterotoxin insensitive.

The recent cloning and characterization of the structural genes for CDT is the first breakthrough in the genetic analysis of *Campylobacter* toxins (97). Pickett et al. used degenerate primers, derived from the DNA sequence of CDT of two *E. coli* strains that were described recently (96, 105), to amplify a fragment from the *C. jejuni* genome coding for CDT. The amplified fragment was used as a probe to clone the complete locus (2.6 kb) of CDT. As in *E. coli*, three structural genes are required for cytotoxin activity: *cdtA*, *cdtB*, and *cdtC*. The presence of these three *C. jejuni* genes allows (low-level) expression of active CDT in a nontoxigenic *E. coli* host. The three proteins are of a size similar to the size of the *E. coli* proteins and probably have similar yet unknown functions, although their amino acid sequences have diverged from those of the *E. coli* CDT. (97). Like *E. coli* CDT, the toxin of *C. jejuni* is strongly hydrophobic, suggesting that one of the proteins is a lipoprotein (96). The *cdtB* gene was detectable by hybridization in all 11 *C. jejuni* strains tested. There was strong relatedness in tests with *C. jejuni* and weaker relatedness with *C. coli* strains. The

HeLa cell assay was more sensitive to bacterial sonicates compared with bacterial culture filtrates as used by Johnson and Lior (46). The sonicates of 20 *C. jejuni* strains tested expressed CDT, of which 18 strains produced high titers of the toxin. In contrast, low titers were found with all 12 tested *C. coli* strains. A putative *cdtB* gene was also detectable by PCR, but not by DNA hybridization (suggesting a low level of homology), in single strains of *C. fetus*, *C. hyointestinalis*, *C. lari*, and *C. upsaliensis*. In summary, the results indicate that heterologous *cdt* genes are present in most or all *Campylobacter* spp. but that CDT expression or activity varies among species.

**Shiga-like toxin.** Only one report exists on a cytotoxin that could be neutralized by Stx antitoxin (82). It was detected in bacterial lysates of 11 of 36 strains of *C. jejuni* and *C. coli*. The toxicity was measured with HeLa cells. Despite the immunological cross-reactivity, no homology was detected between the *stx<sub>1</sub>* gene of *E. coli* and the genome of cytotoxic strains of *C. jejuni*. With this single publication on Stx production by *Campylobacter* spp., the evidence remains anecdotal. The same study also described a cytotoxin for HeLa cells that could not be neutralized; the toxin was probably identical to the 70-kDa cytotoxin discussed above.

**Hemolytic cytotoxins.** It is now recognized that some *Campylobacter* strains are hemolytic: hemolysis was described on blood agar plates on which *C. jejuni* (92% of the strains tested) and *C. coli* strains (22%) were cultivated at 42°C for 4 days (3). Organisms on plates incubated at 37°C were nonhemolytic, as were *C. lari* and *C. fetus* strains (3). The hemolytic activity was observed only with ageing bacteria, suggesting that it could be an intracellular component that is released when bacteria die and lyse. This and most other observations described cell-associated or contact hemolysis.

Alpha hemolysis is rarely observed and appears to be dependent on the pH of the medium. The typical green zone around a colony appeared within 48 h at 37°C on rabbit blood agar plates at pH 6.0 to 6.5, which is lower than that of most *Campylobacter* media (78). Beta hemolysis, producing clear zones on blood plates, was observed with the same strains that produced alpha hemolysis, but only after 6 days of incubation at 37°C or 3 days at 42°C. The zones did not spread but remained in close contact with the colonies (78). Shorter incubation under aerobic conditions with the use of agarose as the solid base in blood plates produced wider, clear hemolytic zones within 24 h in 18 of 20 *C. jejuni* strains (112).

In a contact hemolysis assay, a bacterial suspension is mixed with erythrocytes and centrifuged to enhance the contact between bacteria and cells, so that cell-bound hemolysins can be detected. By using this assay, hemolytic activity was demonstrated in six of eight *C. jejuni* strains tested (95). A more sensitive microplate assay was developed to measure contact hemolysis, which resulted in moderate titers in some *C. jejuni* strains and low titers in 94% of the strains (112). Culture supernatants or bacterial lysates were always nonhemolytic, which further indicates the cell-associated nature of this hemolytic activity.

In an attempt to clone a hemolysin gene from *C. jejuni*, an expression library in *E. coli* was screened for hemolysis. All positive clones contained the same gene on their insert, which coded for a lipoprotein with homology to siderophore-binding proteins. The protein probably functions as an iron-scavenging siderophore in *C. jejuni* and conferred a hemolytic phenotype on *E. coli*, but it is not present in culture filtrates. The gene is present in all six *C. jejuni* strains and in one *C. coli* strain tested (92). This siderophore may be responsible for some or all of the cell-associated beta-hemolysin in *C. jejuni* and *C. coli*, but this has not been verified. It would be interesting to know

whether expression of the siderophore was iron regulated, since it has been reported that contact hemolysin is not iron regulated (95).

Another possible explanation for the cell-associated hemolytic activity is the production of a hemolysin similar to that expressed by enteropathogenic *E. coli*. The gene encoding this enterohemolysin has been cloned recently from the bacteriophage that carried it (7), and the 60-kDa protein shares some of the properties with *Campylobacter* contact hemolysin: *E. coli* enterohemolysin produces small hemolytic zones, is detected in stationary-phase cultures only, and is absent in the culture medium (7, 8). The two different enterohemolysin genes that have been identified are not homologous to known hemolysin genes (7–9, 110). Although hemolytic activity of *Campylobacter* spp. has not been associated with bacteriophages or plasmids, now that genes coding for enterohemolysin have become available (9, 110), it is relatively easy to determine if there is any relatedness.

Secretion of hemolytic activity into the culture medium has also been reported. In one early publication, a cytotoxin also displayed hemolytic activity (75). This cytotoxin, which was toxic to CHO cells, could be neutralized by the anti-hemolysin/cytolysin antitoxin of *V. cholerae*. The hemolytic phenotype was detected in 27 of 60 *C. jejuni* and *C. coli* strains tested and could be induced in nonhemolytic strains by rabbit ligated ileal loop passage (75). Attempts to identify genetic homology to *V. cholerae* cytolysin have not been reported. No hemolytic activity of the other cytotoxins was tested or reported. In one other instance, both heat-labile and heat-stable hemolytic activities were reported in culture filtrates of *C. jejuni* isolated from Scotland or Bangladesh (42). Positive strains (10 of 15) were isolated from patients with dysenteric diarrhea and from those with cholera-like diarrhea (42). Neutralization with anti-cytolysin of *V. cholerae*, was not carried out. The hemolytic activity of two strains was further characterized; hemolytic activity was trypsin sensitive and could be precipitated by ammonium sulfate, indicating its proteinaceous nature. The hemolysins of the two strains differed in temperature sensitivity: one culture filtrate was partially resistant to treatment for 30 min at 100°C, the other filtrate was inactivated by this treatment (42).

These reports on hemolytic cytotoxin production by *Campylobacter* spp. are obviously preliminary, and further work is needed. Secreted hemolytic activity should be tested for cytotoxicity to other cell types, and if specific antiserum becomes available, it can be determined how many different hemolytic cytotoxins exist. Further research will possibly reveal that hemolytic, membrane-damaging cytotoxins also belong to the repertoire of toxins produced by *Campylobacter* spp.

**Hepatotoxin.** Some clinical isolates of *C. jejuni* from watery and bloody diarrhea and one strain from an asymptomatic patient could induce hepatitis in mice (54). A bacterial sonicate fraction, which was toxic to hepatocytes but not CHO or Y1 cells, was isolated from such a *C. jejuni* strain (53, 54). The crude sonicate fraction (but not culture filtrates) caused liver lesions when applied intravenously to mice, similar to the lesions seen after liver colonization by hepatotoxic *C. jejuni* strains (53). These results suggest that some strains have the potential to colonize the liver and cause hepatitis by expressing a hepatotoxin, but the nature and mechanism of this factor remains to be elucidated.

#### Incidence of Cytotoxin and Cytotoxin/Enterotoxin Activity

The incidence of the cytotoxin(s) and of simultaneous enterotoxin/cytotoxin activity was determined in several studies and compared to the clinical manifestations. A clear correla-

tion was found in a study of 20 human isolates, of which 12 were from asymptomatic carriers and 6 were from patients suffering from secretory and inflammatory diarrhea (58). All the strains from asymptomatic carriers were toxin negative, whereas all the strains associated with inflammatory diarrhea were cytotoxic (Vero/HeLa cell cytotoxin) and all the strains causing watery diarrhea were enterotoxigenic. In the last group, one strain was weakly cytotoxic as well (58). A weaker correlation was found for 44 strains isolated from children in Costa Rica (33) who were suffering from watery ( $n = 26$ ) or inflammatory ( $n = 18$ ) diarrhea: in the first group, no cytotoxin-producing strains (but 16 enterotoxigenic strains) were isolated; in the second group, 2 70 kDa cytotoxin-positive strains were found, 1 of which also expressed enterotoxin (33). A higher incidence (45%) of cytotoxicity (probably the 70-kDa cytotoxin as determined with CHO cells) was reported for 22 isolates from patients with inflammatory diarrhea in the United States (93). However, only two strains (9%) had high titers of cytotoxin, and a correlation with the clinical symptoms was absent (93). A total of 60 clinical and 30 animal isolates were compared with 50 isolates from asymptomatic carriers from Bangladesh for cytotoxin production against HeLa cells. Of the clinical isolates, 84% were positive, as were 33% of the animal and 7% of the carrier isolates (2). Cytotoxicity was also measured for Vero cells but a lower percentage of strains was positive. This suggests that, at least in some cases, the 70-kDa cytotoxin, to which Vero cells are insensitive, was detected. The cytotoxicity for Vero cells most probably detected HeLa/Vero cytotoxin (2). In similar studies on the incidence of toxin production by clinical isolates without clinical information, cytotoxin positivity was reported to be 87% for the 70-kDa cytotoxin ( $n = 47$  [36]), 81% as determined with HeLa cells ( $n = 22$  [15]), and 48% for the 70-kDa cytotoxin ( $n = 29$  [91]). In a large study comprising 316 clinical isolates, Vero/HeLa cell cytotoxin and enterotoxin production was determined (45). It was found that 72% of the strains produced the cytotoxin, of which 58% also produced the enterotoxin. Differences in the assay conditions of cytotoxicity may be responsible for the variation in incidence among different studies, and it is not always clear which cytotoxin was determined. However, cytotoxin production seems to be more common in strains isolated from patients with inflammatory diarrhea than in other strains. The incidence of cytotoxin production in *C. jejuni* is higher than in *C. coli*, like the incidence of enterotoxin production (3, 15, 80, 97).

Cytotoxin production was also demonstrated in strains isolated from animal sources, mainly from poultry (76). Different cell lines were tested, and the highest incidence of cytotoxicity was found with CHO and fetal calf lung cells, as opposed to Vero, HeLa, and chicken embryo fibroblast cells. Many isolates (33%) were positive in all cell lines tested, and nontoxigenic strains were not detected (76). In a study comparing the cytotoxicity of human and animal isolates ( $n = 34$  and 22, respectively), the frequency of cytotoxicity was identical in the two groups, as determined by three different CHO cell assays (80).

The significance of cytotoxin production during inflammatory diarrhea was questioned by Perez-Perez et al. (93), since the serum of patients did not neutralize the homologous toxins. However, cytotoxin that is produced in vivo does not have to be immunogenic (see above), and serum containing antibodies against cytotoxin does not have to neutralize the toxin, because functional domains and immunogenic epitopes need not be identical.

In other members of the genus *Campylobacter*, expression of cytotoxins has been reported for four *C. lari* strains of human

origin, two of which were also enterotoxin positive (45), but the nature of the cytotoxin was not further investigated. A CDT activity indistinguishable from *C. jejuni* CDT activity was detected in a *C. lari* strain and in a catalase-negative *Campylobacter* strain (46), as well as in *C. fetus* strains (88). Interestingly, CDT was produced mainly by *C. fetus* strains isolated from calves suffering from diarrhea, as opposed to liver isolates (88). The cytotoxin activity of four of seven *C. fetus* strains as detected by trypan blue staining of CHO cells (38) could also be due to CDT activity. Finally, a cytotoxin was isolated from *C. rectus*, which was formerly known as *Wolinella rectus* and is considered an etiologic agent of periodontal disease. The cytotoxic activity that was present in culture filtrates could be purified to a 104-kDa protein that showed an amino acid distribution similar to that of RTX toxins like *E. coli* hemolysin (HlyA) (34). This finding indicates that a representative of the RTX toxin family is also present in a member of the *Campylobacter* genus.

### Proposal for Standardization and Classification

This review is meant to bring some structure and order to the confusing literature on cytotoxins produced by *Campylobacter* spp. The classification of cytotoxins proposed here is based on the available data without any experimental comparison. It is important to distinguish the different cytotoxins, because some may be of more clinical relevance than others. Ideally, each newly described cytotoxic activity should be compared to a panel of existing "archetypes" of known and characterized cytotoxic strains. Alternatively the development of specific antiserum against CDT, 70-kDa cytotoxin, and Vero/HeLa cell cytotoxin (and other characterized toxins) would enable an easy identification procedure by means of neutralization studies. To verify the classification presented here, it is proposed that (i) cytotoxicity be tested (among other cell types) against HeLa and Vero cells to distinguish between the first two types of cytotoxins; (ii) transcriptional activity of *cdt* be tested in CDT-expressing strains, e.g., by the reversed PCR technique; (iii) Stx-like cytotoxin be identified by neutralization with Stx antitoxin; and (iv) cell-associated hemolytic activity be screened with the genetic probes for the siderophore protein and for *E. coli* enterohemolysin. Secreted hemolysin can be compared to *V. cholerae* cytotoxin, by both antiserum neutralization and genetic methods. The activity of the hepatotoxin seems to be hepatocyte specific and would not easily be confused with other cytotoxins. Any newly identified cytotoxins should be added to this or a better classification system, and nomenclature of cytotoxins should be standardized. Once the mechanisms of cytotoxicity are known, cytotoxins can finally be classified based on their mechanism of action.

### FUTURE DIRECTIONS OF TOXIN RESEARCH

It will be clear that toxin research is not a closed book for *Campylobacter* spp. More data are needed to judge the relevance of enterotoxigenic activity, and the clinical significance of the various cytotoxins is still largely unknown. In the past, the classical methods of biochemical purification did not result in toxins of significant purity and quantity needed for structural and functional analyses. The alternative is to use molecular biology in the *Campylobacter* toxin field. By analogy to other bacterial toxins, a major step forward in toxin analysis can be expected once the structural genes become available. The initial progress has been made with the cloning of the *cdt* genes. The most successful way to clone toxin genes that are not homologous to known toxins is to screen a gene library with



specific antiserum or to functionally screen individual clones. Since bacterial toxins often require multiple genes for production, activation, secretion, and expression, cloning of the structural gene only may not be sufficient. In most cases, however, all of these genes are present at a single locus (although regulation of expression is often contained elsewhere on the genome), allowing toxin expression if the cloned DNA fragment is large enough. To overcome a lack of expression or toxic effects in *E. coli*, a nontoxicogenic *Campylobacter* strain is the host of choice. The standard cloning techniques have now also become applicable to *Campylobacter* research (40). The next phase of toxin research may very well prove that toxin production is one of the components of the pathogenic mechanism of *Campylobacter* spp.

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